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Immunohistochemical Localizations of TGF-β in the Developing Rat Gonads

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ABSTRACT—Beta types of transforming growth factor (TGF-βs) are known to have a variety of types of endocrine, paracrine and autocrine roles in the adult and embryonic tissues. In order to clarify the participation of TGF-β in rat gonadal differentiation, immunohistochemical expression of TGF-β was chronologically studied in perinatal rat gonads. Sprague-Dawley rat gonads from gestational day (GD) 13 to postnatal day (PD) 21 were fixed in Methacarn solution and stained with a polyclonal antibody against native porcine platelet TGF-β in the rabbit by using avidin-biotin complex technique.

Immunohistochemical reactivity to TGF-β was positive in the germ cells of both sexes from GD 13 to PD 21. Moderate and marked staining was seen in male germ cells from GD 16 to PD 21 and in female germ cells from GD 21 to PD 11. Leydig/interstitial cells in male gonads were stained from GD 15, and moderate and marked staining from GD 16 to PD 11. On the other hand, the Sertoli or peritubular cells was not stained during the perinatal period. In contrast to the male gonads, the granulosa and interstitial cells in female gonads were stained on PDs 11 and 21. The Wolffian ducts in males and the Müllerian ducts in females expressed positive but weak reactivity during the perinatal period. The mesonephric tubules were faintly stained from GD 13 to 18.

These results indicate that TGF-β shows discrete cell-specific patterns of expression at various stages of the rat gonadal development and may participate in the rat gonadal development and differentiation.

INTRODUCTION

Beta type of transforming growth factor (TGF-β) was identified and isolated on the basis of its ability to induce anchorage-independent growth of non-transformed cells [7]. TGF-βs, homodimeric 25-kDa peptides, are known to have profound effects on nearly all types of cells, influencing their proliferation and differentiation [for reviews, 28, 29, 32]. After the cloning of cDNA of TGF-β1 [8], five members of TGF-β have been isolated. These are characterized by 70% amino acid identity within the bioactive C-terminal portion. Purified and recombinant proteins of TGF-β1, β2 and β3 have been obtained from various mammalian sources.

In recent years, members of TGF-β play important roles in many different embryonic processes [3, 26]. TGF-βs are mitogenic for mesenchymal and supporting cells such as bone and cartilage but are inhibitory for mitosis in many other cell types. In addition, the TGF-βs regulate differentiation, stimulate extracellular matrix deposition and induce mesoderm formation during early embryogenesis. The *in vivo* localizations of mRNAs for TGF-β1, -β2 and -β3 during mouse embryogenesis have been described in many reports [17, 20, 22, 24, 25, 30, 33]. Pattern of TGF-β mRNA expression changed as development progressed and the expression was found in tissues undergoing morphogenetic alterations. On the other hand, expression of TGF-β proteins to which the mRNAs are translated has also been reported in mouse by using the immunohistochemical technique [13, 27]. TGF-β proteins were detected in many tissues, e.g. bone, kidney, liver and nervous tissue, and were frequently colocalized with the same cell types as the mRNA-expressed cells. In spite of evidences that many tissues showed expressions of TGF-β mRNA and its proteins, little is known about the

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TGF-β protein expression during the gonadal development in rat.

In order to determine the participation of TGF-β proteins in rat gonadal differentiation, immunohistochemical expression of TGF-β was chronologically clarified in the fetal and prepubertal rat gonads from gestational day (GD) 13 to postnatal day (PD) 21.

MATERIALS AND METHODS

Experimental animals

Crj:CD (Sprague-Dawley) rats in 13 to 20 weeks of age were housed in constant temperature ($22 \pm 2^{\circ}C$), relative humidity ($55 \pm 10\%$) and light-dark cycle (lights on 7:00–19:00). The animals fed purina chow and took the tap water *ad libitum*. Cohabitation was done in the evening in the 1:1 basis of male:female. In the next morning, copulation was checked by the presence of sperm in the vaginal smear. The day when sperm-positive smear was found was designated as GD 0, and the day when litter was found was designated as PD 0.

Preparation of tissues for immunostaining

Dams were sacrificed from GD 13 to 21 and neonates on PDs 5, 11 and 21 by carbon dioxide. The gonads and genital ducts dissected from at least three fetuses and pups were fixed in Methacarn solution consisting of methanol, chloroform and acetic acid, 6:3:1 in volume for a few hours to overnight at 4°C. The sexes of fetuses were determined as described by Agelopoulou et al. [2]. Then, the tissues were dehydrated through a series of graded concentrations of ethanol and xylene, embedded in paraffin and sectioned in 5 μm thickness.

Immunohistochemistry

Sections were deparaffinized with xylene and hydrated in decreasing concentrations of ethanol, incubated in 6 M urea (ICN Biomedicals Inc.) at room temperature for 30 min and to block endogenous peroxidases with 0.5% periodic acid (Sigma Chemical Co.) for 15 min. Sections were subsequently rinsed with 10 mM phosphate buffered saline (PBS, pH 7.4, Sigma Co.) for 20 min, blocked non-specific staining with 1.5% normal goat serum in 10 mM PBS including 0.5% casein (Wako Pure Chemical Industries Ltd.) for 20 min and then incubated with avidin and biotin blocking solution (Vector Laboratory Inc.) for 15 min each at room temperature. After that, sections incubated overnight at 4°C with the polyclonal antibody against native porcine platelet TGF-β raised in the rabbit (R&D systems) at 5 μg/ml in 10 mM PBS including 0.5% casein. Dose-response study indicated that this concentration of the antibody gave optimal labelling results. Following this incubation the sections were rinsed with PBS and then treated with 0.5% biotinylated goat anti-rabbit secondary antibody (Vector Lab. Inc. ABC-peroxidase staining kit Elite) diluted in 10 mM PBS containing 0.5% casein for 30 min at room temperature. Sections were again washed in PBS and subsequently incubated with 2% avidin-biotin complex (Vector Lab. Inc. ABC kit Elite) in 10 mM PBS for 60 min at room temperature. Avidin and biotin were prepared at least 30 min before applied to the sections to allow the complex to form. The sections were again washed in PBS, and the bound antibody was visualized with 0.05% 3,3′-diaminobenzidine tetrachloride (Sigma Chemical Co.) in 10 mM Tris-buffered saline (pH 7.2, Sigma Chemical Co.) and 0.01% H_2O_2 for 5 min.

Controls included (a) replacing the primary antibody with normal rabbit serum, (b) using the primary antibody that had been pre-incubated overnight at 4°C with 10 μg/ml porcine TGF-β1 (R&D systems) before this mixture was applied to the section in order to check the specificity of the primary antibody, and (c) omitting the primary antibody to check the specificity of the secondary antibody.

RESULTS

Specificity of antibody

Preparations which were stained with the antibody to TGF-β, and immunoneutralized antibody by pre-incubated with the antigen or with normal rabbit serum were shown in Fig. 1. TGF-β antibody stained the kidney cells of fetal rats on gestational day 18, but the neutralized antibody or

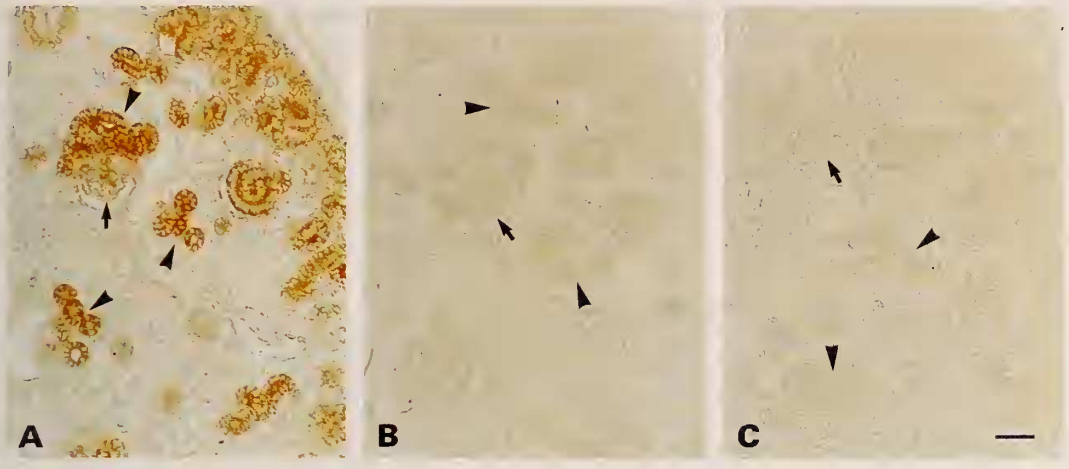

FIG. 1. Demonstration of the staining specificity. The kidneys on GD 18 incubated with the TGF-β antibody (A), with the neutralized antibody (B) or with normal rabbit serum (C). Staining is seen in the Bowman's capsules (arrow) and the collecting tubules (arrow head) of A, but not those of B or C. Bars: 50 μm.

TABLE 1. Immunohistochemical Localizations of TGF-β in the Developing Rat Gonads

	Male							Female						
	Gonad				Duct		MT	Gonad				Duct		MT
	G	S	P	L	W	M		G	Gr	T	I	W	M	
GD 13	+	-			-		+	+				-		+
14	+	-			+/-	-	+	+	-		-	-	-	+
15	+	-		+	+/-	-	+	+	-		-	-	-	+
16	++/+	-		++/+	+/-	-	+	+/-	-		-	-	-	+
17	+++	-		++	-		-	+/-	-		-	-	+/-	+
18	+++	-	-	+++	+		+	+	-		-	-	+	+
19	++/+	-	-	+++	+/-			+/-	-		-		+	
20	++/+	-	-	+++	+/-			+/-	-		-		+/-	
21	++/+	-	-	+++	+			+++	-		-		-	
PD 5	++/+/-	-	-	+++	+/-			+++	-	-	-		+	
11	++/+/-	-	-	+++	+			++/+	++/+	-	++		+	
21	++/+/-	-	-	++/+	-			+	+/-	-	++		-	

The degree of reactivity of the cells or tissues against TGF-β antibody was shown. Grade, -: No detectable, +: Slight but above background levels, ++: Moderate,+++: Marked staining. GD: Gestational day, PD: Postnatal day, G: Germ cell, S: Sertoli/Supporting cell, P: Peritubular cell, L: Leydig cell, W: Wolffian duct, M: Müllerian duct, MT: Mesonephric tubules, Gr: Granulosa/Supporting cell, T: Theca cell, I: Interstitial/stromal cell.
Shade box was shown that the cells or tissues were not found in that day.

normal rabbit serum did not stain any cells. Therefore, these results showed that this polyclonal antibody specifically stained TGF-β-containing cells because the fetal rat kidney was confirmed to contain TGF-β-positive cells in the preceding reports [3, 27, 30].

Immunohistochemical localization

Immunohistochemical expression of TGF-β in the developing rat gonads was summarized in Table 1.

The primordial germ cells and germ cells showed positive reactivity to the antibody from GD 13 to

PD 21 in both sexes. The patterns of development-associated reactivity were different between both sexes; the staining intensity in most of male germ cells was moderate and marked from GD 16 to PD 21 (Fig. 2 A and B), but that in most of female germ cells was marked from GD 21 to PD 11 (Fig. 2 E). The number of male germ cells with positive reactivity was decreased after PD 11. Leydig cells positively stained from GD 15, the time when differentiating Leydig cells were firstly recognized by histochemical examination. The staining intensity and the number of the Leydig cells with positive staining increased with the gonadal development during the perinatal period: marked staining was seen in almost cells from GD 18 to PD 11 (Fig. 2 C). On the other hand, female

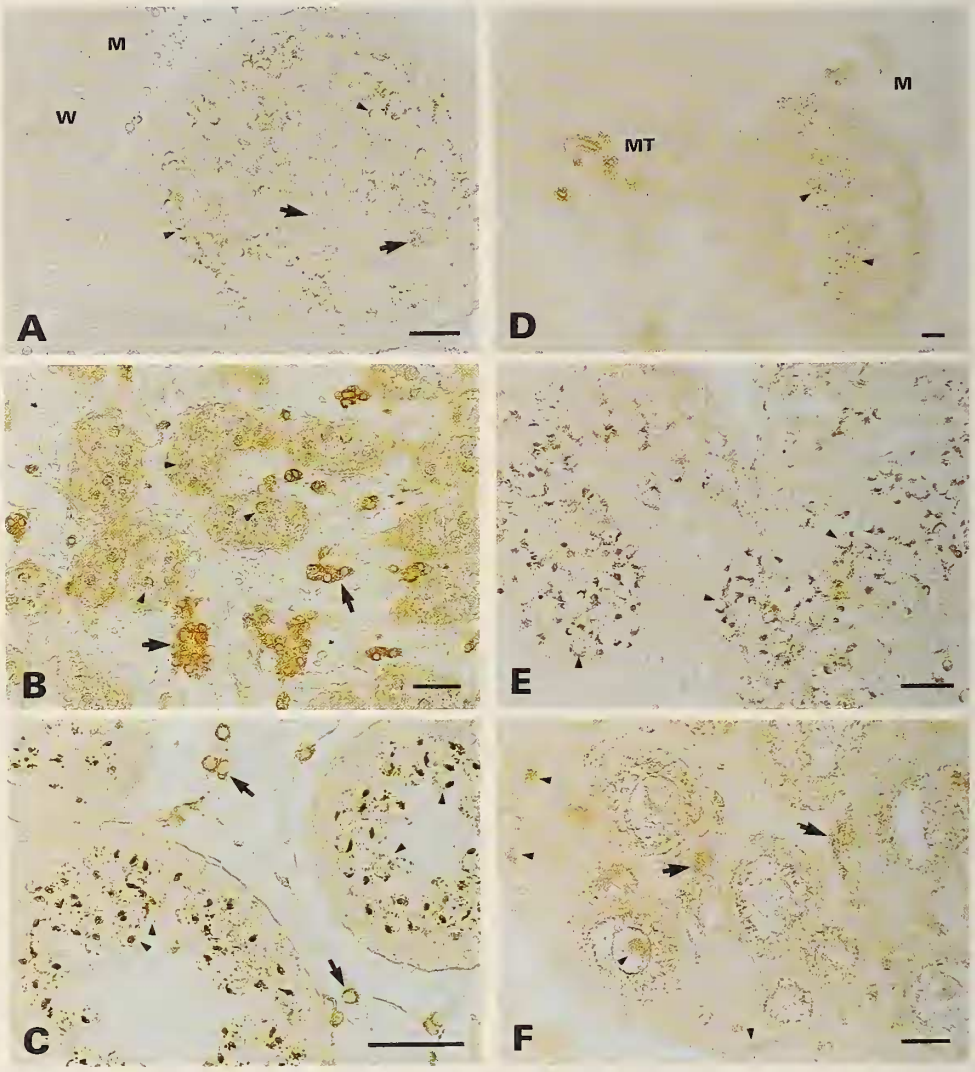

FIG. 2. Immunohistochemical localizations of TGF-β in the developing rat gonads. A, B and C show male gonads on GDs 16 and 21, and PD 11, respectively. D, E and F show female gonads on GDs 18 and 21, and PD 11, respectively. Moderate and marked staining is obtained in the male germ cells (arrow head) and the Leydig/interstitial cells (arrow) during the perinatal periods. Slight to marked staining is obtained in the female germ cells (arrow head) during the perinatal period and moderate staining in the granulosa and interstitial cells (arrow) on PD 11. M: Müllerian duct, MT: Mesonephric tubules, W: Wolffian duct. Bars: 50 μm.

interstitial cells, which were same origin as Leydig cells, did not express positive reactivity during the prenatal period (Fig. 2 D). Positive signs were obtained in most of the interstitial cells on PDs 11 and 21 (Fig. 2 F).

The reactivity of Sertoli and peritubular cells was negative during the perinatal period (Fig. 2 A, B and C). And also the granulosa cells were negative during the fetal period, but became positive on PDs 11 and 21 (Fig. 2 F). Moderate staining was found in most of the granulosa cells on PD 11 but slight staining in some cells on PD 21. The thecal cells which should be recognized after birth were negative during this experimental period.

The expression in Wolffian ducts was slightly positive in males for several days during the perinatal period, but negative in females. In contrast, the Müllerian ducts showed negative expression in males, but slightly positive in females on GD 17 to 20, and on PDs 5 and 11. The mesonephric tubules showed faintly positive expression from GD 13 to 18 in both sexes.

DISCUSSION

The positive staining to anti-TGF-β serum was observed in some types of cells in the gonads during rat prenatal development. Primordial germ cells (PGCs) had already expressed positive staining on GD 13 in both sexes. PGCs migrate from the extraembryonic mesoderm to the germinal ridge and are proliferating during this migrating period, up to GD 13 [19, 21]. Therefore, positive staining in PGCs on GD 13 indicates that TGF-β may be related to the proliferation of PGCs. Male germ cells can not differentiate to spermatogonia until the neonatal life [11, 31]. The results of intensive staining in male germ cells from GD 16 to PD 5 indicate that TGF-β may play roles as a paracrine mediator for functions of the neighboring cells, such as the induction of intermediate filament synthesis [9] for seminiferous tubule formation and the Müllerian-inhibiting substance(s) production in the Sertoli cells [14, 15]. On the other hand, female germ cells expressed the slight/faint reactivity during most of gestation period, but marked reactivity was obtained from GD 21 and PD 5. Female PGCs differentiate to the primary oocytes during the fetal period and the primary follicles are formulated after birth [6, 12]. Therefore, TGF-β may participate in the primary follicle formation in female gonads as well as seminiferous tubule formation in male gonads.

Leydig cells expressed positive reactivity from GD 15 to PD 21. The intensity of staining and number of the cells stained was gradually increased with gonadal development. Leydig cells were morphologically recognized on GD 15 [19 and the present study], and thereafter show the ability of androgen synthesis *in vitro* [10, 23, 34]. Therefore, the positive stainings in these stages indicate that TGF-β may participate in the Leydig cell differentiation, proliferation and steroidogenesis in the fetal gonads. However, TGF-β inhibits steroidogenesis in adult Leydig cells *in vitro* [4, 18]. There may be differences in the regulation of TGF-β to steroidogenesis between the adult and fetal Leydig cells. The interstitial cells in female gonads were stained on PDs 5 and 11. These cells have not yet been known to participate in physiological function of the ovary. Therefore, this expression may indicate that TGF-β in female interstitial cells would act as a paracrine and/or autocrine mediator of neonatal ovarian function.

The reactivity of Sertoli and the peritubular cells was not detected during the perinatal period. This is not consistent with the results that TGF-β is released from the immature Sertoli cells *in vitro* culture [5]. This inconsistency is due to the difference between *in vivo* and *in vitro*. Therefore, TGF-β may not contribute to the differentiation and development of the Sertoli or peritubular cells *in vivo*. On the other hand, the granulosa cells, of which origin is the same as Sertoli cells, positively stained on PDs 11 and 21. TGF-β stimulates the follicle-stimulating hormone (FSH)-induced aromatase activity in granulosa cells [1] and increases in the response to FSH in the granulosa cells [35]. The expression in granulosa cells indicates that TGF-β may participate in the granulosa cell steroidogenesis in immature female rats as well as that in mature rats.

In our separate study [16], we found that inhibin, a member of TGF-β superfamily, may participate in the seminiferous tubule formation, the

Leydig cell steroidogenesis and the physiological regulations of the Sertoli and granulosa cells. This experiment shows that TGF-β may regulate the germ cell proliferation, the Leydig cell differentiation and proliferation, and the granulosa cell steroidogenesis. Together with these results, growth factors may have wide range of roles on the gonadal development in rats. The mechanisms for endocrine, paracrine and/or autocrine functions of TGF-β as well as inhibins on gonadal development remain to be studied.

REFERENCES

1 Adashi EY, Resnick CE (1986) Antagonistic interactions of transforming growth factors in the regulation of granulosa cell differentiation. Endocrinology 119: 1879–1881

2 Agelopoulou JP, Magre S, Patsavoudi E, Jost A (1984) Initial phases of the rat testis differentiation in vitro. J Embryol Exp Morph 83: 15–31

3 Akhurst RJ, Fitzpatrick DR, Gatherer D, Lehnert SA, Millan FA (1990) Transforming growth factor betas in mammalian embryogenesis. Prog Growth Factor Res 2: 153–168

4 Avallet O, Vigier M, Perrard-Sapori MH, Saez JM (1987) Transforming growth factor β inhibits Leydig cell functions. Biochem Biophys Res Commun 146: 575–581

5 Benahmed M, Cochet C, Keramidas M, Chauvin MA, Morera AM (1988) Evidence for a FSH dependent secretion of a receptor reactive transforming growth factor-β-like material by immature Sertoli cells in primary culture. Biochem Biophys Res Commun 154: 1222–1231

6 Byskov AG (1982) Primordial germ cells and regulation of meiosis. In "Reproduction in Mammals. Book 1. Germ Cells and Fertilization" Ed by CR Austin, RV Short, Cambridge Univ. Press, Cambridge, 2nd ed, pp 1–16

7 De Larco JE, Todaro GJ (1978) Growth factors from murine sarcoma virus-transformed cells. Proc Natl Acad Sci USA 75: 4001–4005

8 Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell JR, Assoian RK, Roberts AB, Sporn MB, Goeddel DV (1985) Human transforming growth factor-beta cDNA sequence and expression in tumor cell lines. Nature 316: 701–705

9 Fridmacher V, Locquet O, Magre S (1992) Differential expression of acidic cytokeratins 18 and 19 during sexual differentiation of the rat gonad. Development 115: 503–517

10 Goldstein JL, Wilson JD (1975) Genetic and hormonal control of male sexual differentiation. J Cell Physiol 85: 365–378

11 Gondos B (1977) Testicular Development. In "The Testis Vol 4" Ed by AD Johnson, WR Gomes, Academic Press, New York, pp 1–37

12 Haffen K (1977) Sexual differentiation of the ovary. In "The ovary Vol 1" Ed by I Zuckerman, BJ Weir, Academic Press, New York, pp 69–122

13 Heine UI, Munoz EF, Flanders KC, Ellingsworth LR, Peter Lam H-Y, Thompson NL, Roberts AB, Sporn MB (1987) Role of transforming growth factor-β in the development of the mouse embryo. J Cell Biol 105: 2861–2876

14 Hirobe S, He W-W, Lee MM, Donahoe PK (1992) Müllerian inhibiting substance messenger ribonucleic acid expression in granulosa and Sertoli cells coincides with their mitotic activity. Endocrinology 131: 854–862

15 Josso N, Picard J-Y (1986) Anti-Müllerian Hormone. Physiol Rev 66: 1038–1090

16 Koike S, Noumura T (1993) Immunohistochemical expression of inhibin-α subunit in the developing rat gonads. Zool Sci 10: 449–454

17 Lehnert SA, Akhurst RJ (1988) Embryonic expression pattern of TGF beta type-1 RNA suggests both paracrine and autocrine mechanisms of action. Development 104: 263–273

18 Lin T, Blaisdell J, Haskell JF (1987) Transforming growth factor-β inhibits Leydig cell steroidogenesis in primary culture. Biochem Biophys Res Commun 146: 387–394

19 Magre S, Jost A (1991) Sertoli cells and testicular differentiation in the rat fetus. J Elect Microsc Tech 19: 172–188

20 Manova K, Paynton BV, Bachvarova RF (1992) Expression of activins and TGFβ1 and β2 RNAs in early postimplantation mouse embryos and uterine decidua. Mech Develop 36: 141–152

21 Merchant-Larios H, Taketo T (1991) Testicular differentiation in mammals under normal and experimental conditions. J Elect Microsc Tech 19: 158–171

22 Millan FA, Denhez F, Kondaiah P, Akhurst RJ (1991) Embryonic gene expression patterns of TGF β1, β2 and β3 suggest different developmental functions in vivo. Development 111: 131–144

23 Noumura T, Weisz J, Lloyd GW (1966) *In vitro* conversion of 7-^{3}H-progesterone to androgens in the rat testis during the second half of fetal life. Endocrinology 78: 245–253

24 Pelton RW, Nomura S, Moses HL, Hogan BLM (1989) Expression of transforming growth factor β2 RNA during murine embryogenesis. Development 106: 759–767

25 Pelton RW, Dickinson ME, Moses HL, Hogan BLM (1990) In situ hybridization analysis of TGFβ3 RNA expression during mouse development: com-

parative studies with TGFβ1 and β2. Development 110: 609–620

26 Pelton RW, Moses HL (1990) The transforming growth factor β family of growth regulatory peptides. In "Contributions to Oncology: Oncogenes in Cancer Diagnostics Vol 39" Ed by CR Bartram, K Munk, M Schwab, Karger, Basel, pp 94–114

27 Pelton RW, Saxena BS, Jones M, Moses HL, Gold LI (1991) Immunohistochemical localization of TGFβ1, TGFβ2, and TGFβ3 in the mouse embryo: expression patterns suggest multiple roles during embryonic development. J Cell Biol 115: 1091–1105

28 Rizzino A (1988) Transforming growth factor-β: multiple effects on cell differentiation and extracellular matrices. Develop Biol 130: 411–422

29 Roberts AB, Sporn MB (1991) The transforming growth factor-βs. In "Peptide Growth Factors and Their Receptors I" Ed by MB Sporn, AB Roberts, Springer-Verlag, New York, pp 419–472

30 Schmid P, Cox D, Bilbe G, Maier R, McMaster GK (1991) Differential expression of TGF β1, β2 and β3 genes during mouse embryogenesis. Development 111: 117–130

31 Setchell BP (1978) Development of the testis. In "The Mammalian Testis" Ed by BP Setchell, Paul Elek, London, pp 30–49

32 Sporn MB, Roberts AB (1992) Transforming growth factor-β: Recent progress and new challenges. J Cell Biol 119: 1017–1021

33 Wilcox JN, Derynck R, (1988) Developmental expression of transforming growth factors alpha and beta in mouse fetus. Mol Cell Biol 8: 3415–3422

34 Wilson JD, Lasnitzki I (1971) Dihydrotestosterone formation in fetal tissues of the rabbit and rat. Endocrinology 89: 659–668

35 Ying S-Y, Becjer A, Ling N, Ueno N, Guillemin R (1986) Inhibin and beta type transforming growth factor (TGF-β)have opposite modulating effects on the follicle stimulating hormone (FSH)-induced aromatase activity of cultured rat granulosa cells. Biochem Biophys Res Commun 136: 969–975